

JP-A-2-213

Publication No. H2-213

Published on January 5, 1990

Application No. 63-258117

Priority: October 19, 1987

JP 62-263263

Filing date: October 13, 1988

Applicant: Taiho Pharmaceutical Co., Ltd

SUSTAINED FORMULATION OF BIOLOGICALLY ACTIVE PEPTIDES

Claims

1. A sustained formulation of biologically active peptide, comprising hyaluronic acid or a non-toxic salt thereof as effective ingredient, said formulation being capable of sustaining effects of the biologically active peptide.
2. The sustained formulation of biologically active peptide according to claim 1, wherein the biologically active peptide is adrenocorticotrophic hormone, thyroid stimulation hormone, growth hormone, follicle-stimulating hormone, somatomedin, growth hormone-releasing factor, epidermal growth factor (EGF), hepatocyte growth factor (HGF), somatostatin, prolactin, vasopressin, vasotocin, mesotocin, isotocin, oxytocin, parathyroid hormone, calcitonin, insulin, glucagon, renin, angiotensin, gastrin, secretin, pancreozymin, enterogastrone, parotin, kallikrein, interferon (IFN), interleukin, tumor necrosis factor (TNF), metallothionein, superoxide dismutase, colony forming stimulation factor, tissue plasminogen activator (TNA), or derivatives thereof.
3. The sustained formulation of biologically active peptide according to claim 1 or 2, wherein the biologically active peptide is a growth hormone, calcitonin, elcatonin or insulin.
4. The sustained formulation of biologically active peptide according to claim 1 or 2, wherein the hyaluronic acid or the non-toxic salt thereof has a molecular weight of 500,000 to 3,000,000.
5. The sustained formulation of biologically active peptide according to claim 1 or 2, wherein the formulation is an injection.

Detailed Description of the Invention

(Industrial Field of the Invention)

The present invention relates to a sustained formulation of a biologically active

peptide.

(Background of the Invention)

Most of biologically active peptide formulations are extracted from human or animal organs, purified and finalized into formulations. The available amount is therefore limited, resulting in insufficient supply to patients in need thereof and the cost is high. Most of these biologically active peptide formulations are for injection, and it is very troublesome for a patient needing frequent administration, imposing a heavy burden for them including mental and physical pain.

A biologically active peptide secreted in body have a certain level and rhythm and it is very difficult to adjust the level to the concentration corresponding to the actual rhythm of human body by using currently available injection formulations, and the effects can be kept only for a very short period of time. A sustained injection method has now been developed for biologically active peptide such as insulin. However it requires a patient to always carry and attach a pump to the body, and resultant troubles and pain cannot be denied.

Thus, for the biologically active peptide formulations there is a demand for a formulation which exhibits its effects at an amount of a drug as small as possible, and furthermore, at a low frequency of administration to maintain the level of the drug at a certain level, sustaining the effects for a long period of time.

On the other hand, hyaluronic acid is a biological component contained in vitreous body of eyes, articular liquid, and ligament at a high concentration, and widely existent also in the other tissues. The significance of hyaluronic acid has been recognized as supporting tissue structure, buffer function (our remark: maybe a clerical error due to Kanji characters and would be "interactive effect") against mechanical stimulation (elasticity of skin and lubricity at joints) and controlling *in vivo* diffusion of substances. Aqueous solution of high-molecular hyaluronic acid or a salt thereof exhibits a pattern of non-Newtonian fluid, and behaves as a high-viscosity substance when it is not flowing. Diffusion of chemical substances in the aforementioned solution was known to be slower than in water in general, because hyaluronic acid acts as a high-viscosity medium when it is dissolved in water (J. Physics. (1961) 150, 67-74). However, more detailed studies revealed that for a substance such as glucose, in contrast to the other substances, the diffusion rate becomes several times higher in a hyaluronic acid solution (J. Biological Chemistry, 257, 23, 14134-14135 (1982)). This deceleration or acceleration of diffusion is therefore considered to be due to an interaction between a compound and hyaluronic acid.

Patents relating to a medical formulation containing hyaluronic acid include Japanese Unexamined Patent Application Publications Nos. 58-57319, 00-50922, and 60-84225, in which the above-mentioned buffering effect of hyaluronic acid was utilized in a medical formulation. The inventions of these applications related to an instillation and an agent for protecting biological tissues. Although JP No. 02-129226 discloses a drug releasing system based on the use of the diffusion retardation property of hyaluronic acid, but it discloses or suggests nothing about physiologically active peptides.

(Problems to be Solved)

It is an object of the present invention to provide a formulation of a biologically active peptide formulation giving a considerably prolonged duration.

(Means for Solving the Problem)

The present invention relates to a sustained formulation of biologically active peptide, comprising hyaluronic acid or a non-toxic salt thereof as effective ingredient, said formulation being capable of sustaining effects of the biologically active peptide.

The inventors made various studies and trials on improvement of a formulation of a biologically active peptide, and found that hyaluronic acid adjusted within a certain concentration range remarkably prolonged the duration of the effects of a biologically active peptide, as compared with an administration of the biologically active peptide alone, and finally established the present invention.

The hyaluronic acid and the non-toxic salt thereof used in the present invention has a molecular weight of 200,000 to 5,00,000 (viscosity process), preferably about 500,000 to 3,000,000. The non-toxic salts include salts of alkali metals such as sodium and potassium and salts of alkaline earth metals such as magnesium and calcium.

Particularly preferable in the formulation is sodium salt. Methods of producing hyaluronic acid and salts thereof are disclosed in JP 58-37001 and 58-57319. Hyaluronic acid used in the present invention may be any one for medical use, for example, hyaluronic acid which is purified such that it does not cause any trouble when injected subcutaneously or into biological tissues. The biologically active peptide for the formulation is a peptide having various biological activities *in vivo* at a trace amount and has a molecular weight of about 1,000 to 1,000,000, typically represented by peptide hormone. The biologically active peptides include peptide hormones such as adrenocorticotrophic hormone, thyroid stimulation hormone, growth hormone, follicle-stimulating hormone, somatomedin, growth hormone-releasing factor,

epidermal growth factor (EGF), hepatocyte growth factor (HGF), somatostatin, prolactin, vasopressin, vasotocin, mesotocin, isotocin, oxytocin, parathyroid hormone, calcitonin, insulin, glucagons, renin, angiotensin (I, II and III), gastrin, secretin, pancreozymin, enterogastrone, parotin, and other peptides such as kallikrein, interferon (IFN), interleukin, tumor necrosis factor (TNF), metallothionein, superoxide dismutase, colony forming stimulation factor, tissue plasminogen activator (TNA) or derivatives thereof, including $\alpha^{1-25\text{NH}_2}$ -D-Ser¹-I-Leu⁴-Val²⁵-ACTH (adrenocorticotrophic hormone), $\alpha^{1-25\text{NH}_2}$ -D-Ser¹-I-Leu⁴-Lys¹⁷-Lys¹⁸-Val²⁵-ACTH, elcatonin, β -naphthyl-azo-polystyrene-insulin, poly-N-vinylpyrrolidone-insulin, triacetylinsulin, A₁₁B₂₉-adipoyl-insulin, A₂₉-lysyl-insulin. The aforementioned biologically active peptides may be usually extracted from mammals (human, monkey, sheep, porcine, bovine, rabbit, whale, etc.), fishes (skipjack, salmon, etc.), and birds (chicken, etc.) and purified, or may be synthesized peptides, including semi-synthesized and recombinant peptides. The formulation ratio of the biologically active peptide cannot be determined uniformly, which depends upon each patient needing administration of this peptide, but it may be the conventional amount for a dose clinically used. More specifically, for example, it is desirable that one dose of formulation contains 50 to 100 international units of follicle-stimulating hormone, 10 to 30 units of vasopressin, 1 to 5 units of oxytocin, 40 to 200 units of calcitonin, 10 to 50 units of elcatonin, 20 to 100 units of insulin, 1 to 3 USP units of glucagon, 0.1 to 0.5 ml of gastrin, 30 to 100 secretin units of secretin, 1 to 5 mg of parotin, 20 to 50 units of kallikrein, 1,000,000 to 5,000,000 units of interferon β . Hyaluronic acid and a non-toxic salt thereof preferably have a formulation ratio of 0.1 to 10 %. It must be noted that a formulation ratio below 0.1% cannot give an effective sustaining effect and a formulation ratio over 10% makes it difficult to inject the formulation subcutaneously, and may cause a problem due to residual hyaluronic acid in tissues after injection. Within a range of 0.1 to 10%, the sustaining effect is prolonged according to the formulation ratio. A more preferable formulation ratio is about 3 to 7%.

The most preferable mode of administration of the sustained formulation of the present invention is a parenteral administration. Conventional route of administration of a biologically active peptide is an injection, particularly a subcutaneous injection. Since the sustained formulation of the present invention has a high viscosity, it is desirable to use a product in which the sustained formulation has been put into a syringe under a germfree condition at the time of manufacturing, although a physician or a patient may suck up the sustained formulation by a syringe from an ampoule and use it. The formulation for injection can be prepared in accordance with a conventional

method known for preparing a formulation for injection. Considering the high viscosity of the hyaluronic acid solution, however, it is important to avoid contamination of air bubbles in the formulation. The air bubbles can be eliminated by introducing the solution or suspension of the formulation into an ampoule or a syringe and deaerating it. The methods for deaeration include centrifugation (3,000 rpm for about 15 minutes) and vacuum. Various additives conventionally used in the art can be added to this sustained formulation of the present invention. The exemplary additives include a local anesthetic, a pH adjuster, an antioxidant, a solubilizing agent and an isotonic agent.

(Examples)

The examples of the present inventions are shown hereinafter.

Example 1 Production of a calcitonin subcutaneous injection

Eight MRC units of porcine calcitonin (Aromour Pharmaceutical Company Ltd.) were dissolved in 1ml of saline and sodium hyaluronate was added. After fully dissolved, the solution was transferred to an ampoule, centrifuged (3000 rpm, 15 min.) for deaeration and the ampoule was sealed to prepare the calcitonin subcutaneous injection.

Porcine calcitonin	8 MRC units
Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Per ampoule	1 ml

Example 2

An insulin subcutaneous injection having the following composition was prepared as described in Example 1.

Porcine insulin	5 units
Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Per ampoule	1 ml

Example 3

A human growth hormone subcutaneous injection having the following composition was prepared as described in Example 1.

Human growth factor	1 units
Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Per ampoule	1 ml

Example 4

An elcatonin subcutaneous injection having the following composition was prepared as described in Example 1.

Elcatonin	8 units
Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Per ampoule	1 ml

Example 5

The ampoule was replaced with a syringe and a syringe containing an insulin subcutaneous injection having the following composition was prepared as described in Example 1.

Porcine insulin	5 units
Sodium hyaluronate	30 mg
Benzyl alcohol	10 mg
Distilled water for injection	Appropriate amount
Per syringe	1 ml

Example 6

The ampoule was replaced with a syringe and a syringe containing a human growth factor subcutaneous injection having the following composition was prepared as described in Example 1.

Human growth factor	1.5 units
Sodium hyaluronate	30 mg
Distilled water for injection	Appropriate amount
Per syringe	1 ml

(Results of Pharmacological tests)**1. Sustaining Effects (Insulin)**

Subcutaneous injections having the following compositions were prepared and used for the experiments.

Comparative Example 1

Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Total	1 ml

Comparative Example 2

Porcine insulin	5 units
Saline for injection	Appropriate amount
Total	1 ml

The injections of Example 2 and the above-identified injections were administered on the backs of male Wistar rats of 8 weeks old, which had been starved for 18 hours, at a dosage of 1 ml/kg. At regular time intervals about 0.4 ml of blood was collected and the blood insulin levels were determined by an enzyme immunoassay using Insulotech-Mochida Kit (Mochida Pharmacy) (Hormone & Rinsho, Vol.26, page 283, 1978). Additionally, blood glucose was also determined under the similar conditions. The results were shown in Table 1.

Table 1

Formulation	Blood insulin level after administration (μ unit/ml)							
	Before administration	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr
Comparative Example 1	2	6	5	4	3	3	5	3
Comparative Example 2	5	1563	162	29	14	11	10	10
Example 2	4	133	85	83	69	41	25	17

Formulation	Blood glucose level after administration / Blood glucose level before administration (%)							
	Before administration	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr
Comparative Example 1	100	99	100	97	105	88	81	85
Comparative Example 2	100	44	38	73	86	85	86	90

Example 2	100	60	55	48	51	48	57	75
-----------	-----	----	----	----	----	----	----	----

For Comparative Example 1 sodium hyaluronate did not affect on the blood insulin level. For Comparative Example 2 high insulin level was observed immediately after administration, which was disappeared by 4 hours post-administration. This is a phenomenon that is conventionally observed by subcutaneous insulin administration and the blood insulin level would not be sustained after an appearance of a transient peak. On the other hand, for the injection of Example 2 the peak of high level of blood insulin, which was observed for Comparative Example 2 immediately after insulin administration, was not observed. Instead the blood insulin level was maintained at an appropriate level over 10 hours. About 2.5-fold prolonged duration was confirmed as compared to the formulation without hyaluronate (Comparative Example 2).

2. Sustaining and Enhancing Effects (Calcitonin)

A subcutaneous injection having the following composition was prepared and used for the experiment.

Comparative Example 3

Porcine calcitonin	5 units
Saline for injection	Appropriate amount
Total	1 ml

The Injections of Example 1, Comparative Example 2 (37) and the above-identified injections were administered on the backs of male Wistar rats of 8 weeks old, which had been starved for 18 hours, at a dosage of 1ml/kg. At regular time intervals about 0.4ml of blood was collected and the blood calcium levels were determined by orthocresolphthalein complexone method (Analytical Biochemistry, Vol.18, p.512, 1967) using Calcium Test Wako Kit (Wako Junyaku). The results are shown in Table 2.

Table 2

Formulation	Blood calcium level after administration (mg/dl)							
	Before administration	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr
Comparative Example 1	9.38	9.56	9.54	9.41	9.41	9.06	8.98	8.87
Comparative Example 3	9.48	7.14	6.49	7.94	9.23	8.91	9.02	9.05
Example 1	9.47	6.96	6.12	5.60	5.71	7.99	8.77	8.82

Calcitonin is a biologically active peptide of 32 amino acids, which is involved in the homeostasis of calcium metabolism.

For Comparative Example 1 sodium hyaluronate did not affect on the blood calcium level. For Comparative Example 3 calcitonin exhibited an effect reducing the blood calcium level immediately after administration, but the reducing effect was low and the duration of the effect was short. For Example 1 the reducing effect on the blood calcium level was high and the duration of the effect was longer. Thus the formulation of the present invention that contains a mixture of calcitonin and sodium hyaluronate exhibited a prolonged duration of the effect, as compared to the administration of calcitonin alone.

Another experiment was conducted to confirm the enhancing effect of calcitonin in the injection of the present invention using the following protocol.

With the similar procedures used in the experiments for prolonged duration a subcutaneous injection containing 0, 1, 2, 4, 8 or 12 MRC units of porcine calcitonin was prepared and administered to rats. The reduced blood calcium level due to the calcitonin administration was determined and the area under the reduced blood level curve was calculated for each dose of calcitonin. The results were shown in Figure 1.

The followings could be understood from Figure 1. For example, 5.6 MRC units/kg of porcine calcitonin was required for obtaining 10mg·hr/dl of the area under the reduced blood level curve for administration of porcine calcitonin alone. In contrast, for administration in combination with hyaluronate only 1.4 MRC units/kg was required, which indicated that the required amount was sufficient at about 1/4 of the amount required for the administration of porcine calcitonin alone.

3. Sustaining and Enhancing Effects (elcatonin)

A subcutaneous injection having the following composition was prepared and used for the experiment.

Comparative Example 4

Elcatonin	8 MRC units
Saline for injection	Appropriate amount
Total	1 ml

The injections of Example 4 Comparative Example 1 and the above-identified injections were administered on the backs of male Wistar rats of 8 weeks old, which had been starved for 18 hours, at a dosage of 1 ml/kg. At regular time intervals about 0.4 ml of blood was collected and the blood calcium levels were determined by orthocresolphthalein complexone method (Analytical Biochemistry, Vol.18, p.512, 1967) using Calcium Test Wako Kit (Wako Junyaku). The results are shown in Table 3.

Table3

Formulation	Blood calcium level after administration (mg/dl)							
	Before administration	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr
Comparative Example 1	9.61	9.68	9.69	9.64	9.51	9.28	9.49	9.64
Comparative Example 4	9.70	7.48	7.01	6.65	6.30	7.78	8.96	9.28
Example 4	9.33	7.16	6.68	6.10	5.75	6.38	6.44	6.56

Elcatonin is a derivative of calcitonin in which the disulfide bond in the calcitonin molecule was replaced with ethylene bond and which is involved in calcium metabolism. For Comparative Example 1 sodium hyaluronate did not affect the blood calcium level. For Comparative Example 4 elcatonin exhibited a much more stronger reducing effect on the blood calcium level immediately after the administration as compared to the effect of calcitonin, but the effect disappeared 10 hours after the administration. As can be seen in Example 4, when elcatonin was administrated in combination with sodium hyaluronate, the reducing effect on the blood calcium level was sustained. Thus, the formulation of the present invention, which contains a mixture of elcatonin and sodium hyaluronate, prolonged the duration of the effect.

With the similar procedures used in the experiments for prolonged duration a subcutaneous injection containing 0, 1, 2, 4, 8 or 12 MRC units of elcatonin was prepared and administered to rats. The reduced blood calcium

level due to the elcatonin administration was determined and the area under the reduced blood level curve was calculated for each dose of calcitonin.

The results were shown in Figure 2. From Figure 2 the followings can be appreciated. For example, 1.9 MRC units/kg of elcatonin was required for obtaining 10 mg·hr/dl of the area under the reduced blood level curve for administration of elcatonin alone. In contrast, while for administration of elcatonin in combination with sodium hyaluronate only 1.0 MRC units/kg was required, which indicated that the required amount was sufficient at about 1/2 of the amount required for administration of elcatonin alone.

4. Sustaining Effects (human growth hormone)

A subcutaneous injection having the following composition was prepared and used for the experiment.

Comparative Example 5

Human growth hormone	1 unit
Saline for injection	Appropriate amount
Total	1 ml

The injections of Example 3 Comparative Example 1 and the above-identified injections were administered on the backs of male Wistar rats of 8 weeks old, which had been starved for 18 hours, at a dosage of 1 ml/kg. At regular time intervals about 0.4 ml of blood was collected and the blood human growth hormone levels were determined by radioimmunoassay. The results are shown in Table 4.

Table 4

Formulation	Human growth hormone level in blood after administration (ng/dl)							
	Before administration	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr
Comparative Example 1	-	-	-	-	-	-	-	-
Comparative Example 5	-	326.7	196.5	39.8	2.2	-	-	-
Example 3	-	23.2	26.6	26.1	25.6	17.1	14.0	10.6

For Comparative Example 1 sodium hyaluronate did not affect on the blood calcium level.

For Comparative Example 5 a high level of human growth hormone was observed immediately after administration, but it disappeared 4 hours (our remark: 8 hours?) post-administration. In contrast when sodium hyaluronate was administered together as the formulation of Example 3, the human growth hormone was remained as long as 12 hours after the administration, which clearly show the formulation was a sustained type of injection. The normal blood level of human growth hormone for adult man is understood to be maintained at 20 ± 0.4 ng/ml. Since the source of human growth hormone formulation is currently limited, there are many attempts to obtain a high effect at an amount as lower as possible. For example, there is an attempt to divide the number of doses (Chiryogaku, 9, (2), 264, 1982). The formulation of the present invention is able to maintain blood level of human growth at a certain level for a long term.

Reference Example (Tegafur)

A subcutaneous injection having the following composition was prepared and used for the experiment.

Comparative Example 6

Tegafur	10 mg
Sodium hyaluronate	50 mg
Saline for injection	Appropriate amount
Total	1 ml

Comparative Example 7

Tegafur	10 mg
Saline for injection	Appropriate amount
Total	1 ml

The above-identified injections were administered on the backs of male Wistar rats of 8 weeks old, which had been starved for 18 hours, at a dosage of 1ml/kg. At regular time intervals about 0.4 ml of blood was collected and the blood Tegafur levels were determined by liquid chromatography. The results are shown in Table 5.

Table 5

	5-FU level after administration (mg/ml)							
Formulation	Before	1 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12hr

	administration							
Comparative Example 6	-	18.34	17.78	14.24	11.65	3.79	3.94	2.94
Comparative Example 7	-	15.52	17.47	14.83	11.82	4.14	3.66	3.16

Tegafur is an anti-malignant tumor agent having a chemical name of 1-(2-tetrahydrofuryl)-5-fluorouracil, which releases 5-fluorouracil (5-FU).

As can be seen in Comparative Example 7, the blood level of Tegafur was relatively high until 6 hours post-administration for administration of Tegafur alone, and after 8 hours a lower level was sustained. As can be seen in Comparative Example 6, the sustaining effect was not observed for the combined administration of Tegafur and hyaluronate.

Brief Description of the Drawings

Figures 1 and 2 show the relationship between calcitonin dose and elcatonin dose and the areas under the reduced calcium blood level curves, respectively.

Our remarks:

Figure 1

The longitudinal axis represents the area under the reduced calcium blood level curve (mg·hr/dl). The horizontal axis represents the dose of porcine calcitonin (MRC unit/ml/kg). The upper curve corresponds to administration of a combination of calcitonin and hyaluronate. The lower curve corresponds to administration of calcitonin alone.

Figure 2

The longitudinal axis represents the area under the reduced calcium blood level curve (mg·hr/dl). The horizontal axis represents the dose of elcatonin (MRC unit/ml/kg). The upper curve corresponds to administration of a combination of elcatonin and hyaluronate. The lower curve corresponds to administration of elcatonin alone.